

**Study Protocol: Hemoglobin desaturation and red
blood cell adhesion: A potential therapeutic target
in Sickle Cell Disease**

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Significance/ Rationale/ Originality: Sickle Cell Disease (SCD) results from the cumulative and deleterious effects of polymerized deoxygenated sickle hemoglobin (deoxy HbS) on red blood cell (RBC) red-ox balance, membrane integrity, and function. Abnormal adhesion to endothelial cells by HbS-containing RBCs (S-RBCs), recognized decades ago², is a critical component in the complex pathophysiology of SCD, which comprises hemolysis, vasculopathy, inflammation, oxidative stress and thrombophilia^{2,3}. Historically, techniques used to quantify cellular adhesion to endothelial and sub-endothelial components, such as laminin (LN) and fibronectin, have been labor-intensive, time-consuming, and not well-suited for longitudinal analyses during routine clinical care^{2,4,5}. To address this, we developed a novel microfluidic assay, the SCD Biochip, which allowed rapid, preprocessing free, serial, and standardized interrogation of cellular adhesion in whole blood, under normoxic (the SCD Biochip) or hypoxic (the Hypoxia Biochip) conditions^{6,7}. The Hypoxia Biochip incorporates an inner gas-permeable tubing and an outer gas-impermeable tubing as a micro-gas exchanger, through which blood is exposed to hypoxic conditions (5%CO₂/ 95% N₂), before analysis on the Biochip. Using the SCD Biochip, we described an association between RBC adhesion and 1) RBC phenotype (HbAA, HbSS or HbSC); 2) hemolysis^{6,8}; and, in HbSS, 3) disease severity⁹, and 4) treatment with hydroxyurea or blood transfusions⁵. Further analysis of S-RBC adhesion on the 'Hypoxia Biochip' revealed subjects with or without increased adhesion following exposure to hypoxia in vitro: hypoxia enhanced adhesion, HEA or non-HEA, respectively. HEA S-RBCs associated with a more severe clinical phenotype (lower HbF, evidence for more hemolysis, and a higher WBC)¹. We have preliminary data that HEA S-RBCs are more common in male subjects with priapism, which is typically nocturnal.

WBCs are quantitatively (often elevated) and qualitatively (often pro-inflammatory) abnormal in SCD. Monocytes and neutrophils²⁵⁻²⁷ are increased in number, abnormally activated, and associated with adverse outcomes in SCD²⁸. The number of abnormally adhesive leucocytes increase during vaso-occlusive crises. Published reports from Dr. David Zidar and colleagues have described clinically distinct activated monocyte phenotypes in samples from patients with inflammatory conditions, including an expansion of CD14DimCD16+ 'patrolling' monocyte and a CD14+CD16+ 'inflammatory' monocyte, relative to the conventionally predominant 'classical' monocytes, CD14+CD16-²⁹. Activated patrolling and inflammatory monocytes express high levels of procoagulant tissue factor and CX3CR1 (the fractalkine receptor)³⁰. The fractalkine receptor has been reported to mediate integrin-independent leukocyte adhesion, and its expression can be induced in the endothelium by inflammatory cytokines³¹.

Nocturnal hemoglobin desaturation (NHD) is common, treatable, and is associated with adverse outcomes in children and young adults with SCD^{10,11}. NHD, defined by one group as <90% oxygen saturation of Hb (SpO₂) for >10% of sleep time, was diagnosed in over one-third of children and adolescents with SCD¹², and, by us, in more than one-quarter of adults with HbSS, independent of conventionally diagnosed obstructive sleep apnea (OSA)¹³. Prolonged hemoglobin desaturation may be harmful since only deoxy HbS polymerizes. In vitro, hypoxia upregulates endothelial adhesion molecules, such as VCAM-1, ICAM-1 and P-selectin, which are pathogenic in SCD¹⁴⁻¹⁶. The etiology of NHD without OSA is not known and may include ventilation perfusion mismatch and/or shunting due to pulmonary vascular malformations¹⁷. In children and adolescents, NHD associated with an increased risk of CNS events (e.g., stroke and cerebral vasculopathy), pain, enuresis, and priapism^{10,11,18}. In SCD, reticulocytosis, hemolysis¹⁹, lower total Hb, and lower HbF were seen in NHD¹³, and NHD has been pathophysiologically linked to autonomic dysfunction, WBC activation and adhesion, and hypercoagulability²⁰⁻²². Of note, treatment of SCD with hydroxyurea has been reported to improve day- and night-time SpO₂²³.

Likewise, post-exercise hypoxia, although lesser explored, was found to affect 44.7% children and adolescents. This was defined as a decrease in SpO₂ by $\geq 3\%$ after a 6-minute walk test (6MWT)¹². This group reported an association between reduced 6MWT distance with severe hemolysis. Additionally, in children with SCD, decrease in 6MWT distance correlated with silent infarcts, severe anemia, lower hemoglobin F and low red cell deformability^{32, 33}.

The median life-expectancy of people with SCD has risen dramatically in the last 40 years. One consequence of this is an expanding young adult population with comorbidities that have not yet been well characterized. We propose to examine baseline hypoxia, post-exercise hypoxia and NHD in this modern adult SCD population. We will obtain clinical histories, baseline laboratory values and night-time SpO₂ levels. We will exploit our ability to identify activated monocyte subtypes in patients with HbSS with or without comorbid Hb desaturation, in order to better understand deleterious inflammatory consequences that may arise from Hb desaturation at baseline, with exercise, or during sleep. We will measure RBC adhesion at baseline (SCD Biochip) and when exposed to hypoxia in vitro (Hypoxia Biochip) in patients with SCD. Where possible, we will repeat these measurements ~2 months after initial testing in all subjects to assess the impact of therapeutic oxygen in subjects with NHD.

Hypotheses: We hypothesize that disease activity and RBC adhesion will be greater in subjects with concomitant HbSS and baseline hypoxia, exertional hypoxia, or NHD, due to RBC membrane damage from prolonged hypoxia in vivo. Successful treatment with therapeutic oxygen, at baseline, with exercise, or during sleep, may decrease RBC adhesion in vitro.

1. Subjects with Hb desaturation at baseline, with exertion, or during sleep (NHD), compared to those without, will have increased disease activity (exertional or nocturnal symptoms, priapism, WBC activation, reticulocytosis, and/or hemolysis).
2. S-RBCs from subjects with clinical Hb desaturation at rest, with exertion, or during sleep, compared to those without, will have increased adhesion at baseline and when exposed to hypoxia in vitro (HEA).
 - 2.1 Treatment of baseline hypoxia, exertional hypoxia, and/or NHD with supplemental oxygen will decrease S-RBC adhesion and HEA, and may decrease symptoms, especially night-time symptoms.

In SCD, exertional hypoxia and NHD are common, treatable, and associated with bad outcomes in children and young adults^{15,16}. The median life-expectancy of SCD has risen dramatically in the last 40 years. One consequence of this is an expanding young adult population in whom the comorbidities are not yet fully characterized. The prevalence, clinical consequences, and treatment outcomes of exertional hypoxia and NHD are poorly described in adults with SCD. Therefore, it is important to identify and better understand any clinically significant hypoxia (during exercise or sleep or at rest) in this expanding adult population.

We will study whether RBC adhesion at baseline and when exposed to hypoxia in vitro is significantly increased in adult HbSS patients with baseline hypoxia, exertional hypoxia or NHD due to RBC membrane changes arising from prolonged in vivo exposure to hypoxia, which may be mitigated by oxygen therapy.

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2.1 Treatment of baseline hypoxia, exertional hypoxia, and/or NHD with supplemental oxygen will decrease S-RBC adhesion and HEA, and may decrease symptoms, especially night-time symptoms.

Specific Aims:

Specific Aim 1: To evaluate for resting hypoxia, exertional hypoxia or NHD, and its clinical associations, in adults with HbSS.

Specific Aim 2: To examine baseline RBC adhesion and HEA in vitro in adults with HbSS, with and without resting or exertional hypoxia or NHD.

Specific Aim 3: To examine serial changes in S-RBC adhesion at baseline and with hypoxia in vitro, in adults with HbSS and resting or exertional hypoxia or NHD, before and after therapeutic intervention with oxygen.

STUDY PROCEDURES AND GUIDELINES

1) Specific Aim 1: To evaluate for resting hypoxia, exertional hypoxia or NHD, and its clinical associations, in adults with HbSS.

1.1 Study Design/ Overview: In this prospective cross-sectional study, we will approach 100 adults with HbSS (with an estimated active pool of 40-50 adult subjects with HbSS), at clinical baseline (>3 weeks from crisis or transfusion) who, as part of clinical care, will undergo resting SpO₂ testing, nocturnal pulse oximetry and 6MWT. Clinical history, treatment history, and health care utilization for the last 3 years will be recorded, with particular attention to night-time symptoms, including the presence of night time pain, priapism, or early morning headaches. Prior echo-cardiogram data will be recorded, including bubble contrast results, performed by us as a screen for right to left shunts, which are common in adults with HbSS (Hambley, Little et al, under submission)).

1.2 Sample size justification: We estimate that at least one quarter of adults with HbSS in our clinic have NHD, independent of OSA (unpublished, and ¹³). As above, will recruit 40-50 subjects with HbSS, a traditional estimate required for feasibility trials²⁴. We project that a minimum of 10-15 subjects will have NHD, which has been sufficient in our earlier work to yield insights about symptoms and disease severity¹. This study is a pilot effort to estimate the prevalence of exertional hypoxia in adults with SCD.

1.3 Study procedure: We will test resting SpO₂ and order night-time oximetry testing as well as 6MWT in subjects with HbSS who are not on oxygen therapy. We have been ordering night time oximetry as a routine clinical test (VirtuOx pulse oximetry device, FDA and Medicare approved), due to the frequent finding of NHD (seen in 13/24 subjects with HbSS tested over 12 months) and anecdotal improvement in symptoms with oxygen therapy in the approximately 15-20 patients already on therapy. Lab data (CBC, reticulocyte count, LDH and serum chemistries), clinical history, prior screening echocardiogram, and nocturnal symptoms will be obtained from consented subjects, and will be entered into REDCap.

1.4 Study Analysis: We will correlate resting Hb saturation, nocturnal Hb saturation (both as a continuous and as a categorical variable, e.g. with or without Medicare eligibility for night-time oxygen), post exercise Hb saturation and 6-minute walk distance with clinical phenotype (e.g. patient age, evidence for hemolysis, comorbidity profile, health care utilization, echo findings, and treatment history), using univariate and multivariate models.

1.5 Expected outcomes: We predict a more severe clinical phenotype in subjects with HbSS and clinically significant hypoxia (resting or post exercise or sleep), compared to those without.

2) Specific Aim 2: To examine baseline RBC adhesion and HEA in vitro in adults with HbSS, with and without resting or exertional hypoxia or NHD.

2.1 Study Design: Cross-sectional study to evaluate baseline RBC adhesion and HEA, in vitro, in 40-50 adults with HbSS, with or without clinically significant hypoxia.

2.2 Sample size justification: 10-15 patients are likely to have NHD, and up to 30 are not. This sample size previously yielded interpretable data in our analyses of NHD, and of adhesion and HEA^{1,13}. This study is a pilot effort to estimate the prevalence of exertional hypoxia in adults with SCD.

2.3 Study procedure: S-RBC adhesion to LN on the SCD and Hypoxia Biochips will be quantitated, using <400 μ L surplus whole blood in EDTA, obtained during routine clinical care (as published previously^{1,6-8}), at the clinic visit immediately prior to night-time oximetry and 6MWT. Fluorescent Activated Cell Sorting (FACS) following incubation with antibodies to CD14, CD16, and CX3CR1 will be performed on 3-400 μ L of surplus whole blood.

2.4 Study Analysis: Simple t-tests will be used to compare RBC adhesion, HEA to LN and monocyte activation in patients with clinically significant hypoxia and those without any hypoxia.

2.5 Expected outcomes: We predict that patients with clinically significant hypoxia, compared to those without, will have increased RBC adhesion at baseline and with hypoxia in vitro (HEA) as well as increase monocyte/ WBC activation.

3) Specific Aim 3: To examine serial changes in S-RBC adhesion at baseline and with hypoxia in vitro, in adults with HbSS and resting or exertional hypoxia or NHD, before and after therapeutic intervention with oxygen.

3.1 Study Design/ Overview: A prospective, observational and interventional feasibility study will be initiated during this proposal. Paired samples (baseline and ≥ 6 weeks, with or without oxygen therapy) from treated and untreated patients, with and without any hypoxia respectively, will be examined for changes in S-RBC adhesion, HEA, and night-time symptoms.

3.2 Sample Size Justification: This is a pilot study. We estimate that 10-15 patients will have NHD. But, this study is a pilot effort to estimate the prevalence of exertional hypoxia in adults with SCD. Paired analyses will yield novel insights about the impact of oxygen therapy on RBC adhesion, at baseline and HEA in vitro, in treated patients with Hb desaturation.

3.3 Study Procedure: As per clinic routine, patients with HbSS and resting hypoxia or exertional hypoxia or NHD who fulfill Medicare criteria for oxygen supplementation (5 minutes with SpO₂<89%) will be prescribed oxygen. In our experience, the interval from testing to initiation of therapy is approximately 2 weeks. At ~ 2 months after initial testing (and ≥ 6 weeks on treatment, if needed), all subjects will be re-evaluated for night-time symptoms, RBC adhesion (with and without hypoxia in vitro), and clinical history obtained at routine clinic visits. We will repeat Hb saturation testing at this point to assess adherence to therapy.

3.4 Study Analysis: Paired t-test on S-RBC adhesion to LN and HEA before and after oxygen therapy, in subjects with and without clinically significant hypoxia. Night-time symptoms, WBC activation and lab results will also be examined for any suggestive changes.

3.5 Expected outcomes: S-RBC adhesion and HEA will decrease following ≥ 6 weeks of oxygen therapy in subjects with hypoxia and will not change over the same interval in subjects without hypoxia.

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